RALF, a 5-kDa ubiquitous polypeptide in plants, arrests root growth and development

Gregory Pearce, Daniel S. Moura, Johannes Stratmann, and Clarence A. Ryan, Jr.*†

Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340

Contributed by Clarence A. Ryan, Jr., August 8, 2001

A 5-kDa polypeptide was isolated from tobacco leaves that induced a rapid alkalinization of the culture medium of tobacco suspension-cultured cells and a concomitant activation of an intracellular mitogen-activated protein kinase. An N-terminal sequence was obtained, and a cDNA coding for the 49-aa polypeptide was isolated from a tobacco cDNA library. The cDNA encoded a preproprotein of 115 amino acids that contained the polypeptide at its C terminus. A search among known expressed sequence tags revealed that genes encoding *R*apid *AL*kalinization *F*actor (RALF) preproproteins were present in various tissues and organs from 16 species of plants representing 9 families. A tomato homolog of the polypeptide was synthesized and, when supplied to germinating tomato and *Arabidopsis* seeds, it caused an arrest of root growth and development. Although its specific role in growth has not been established, the polypeptide joins the ranks of the increasing number of polypeptide hormones that are known to regulate plant stress, growth, and development.

Polypeptide signals are important receptor-mediated intercellular messengers (hormones) that regulate defensive and developmental processes in plants (1). However, only a few polypeptide signals have been identified, mainly due to the enormous efforts required to either isolate them from plant tissues or identify their genes through mutational analyses.

We developed an assay to identify polypeptide signals that interact with cell-surface receptors of suspension-cultured cells to cause a measurable increase in the pH of the culture medium (2). With this assay, two 18-aa systemins were isolated from tobacco leaves that were found to be derived from the same polyprotein precursor (2). During the purification of the tobacco systemins, we identified another polypeptide component that, at low nanomolar levels, caused the rapid alkalinization of the medium of the tobacco suspension-cultured cells. We named the component the *R*apid *AL*kalinization *F*actor, RALF.

Herein we report the purification of RALF and its identity as a 5-kDa polypeptide. A precursor cDNA was isolated from a tobacco leaf library that coded for a 115-aa preproprotein that has the RALF sequence at its C terminus. Whereas other known plant polypeptide hormone precursor genes have been identified in only a few species, genes encoding the RALF precursor were found throughout the plant kingdom, with the RALF polypeptide regions being highly conserved. The properties of RALF suggest that it is a powerful receptor-mediated signal, processed from a larger precursor, that may have a role in regulating growth and development.

Materials and Methods

Alkalinization Assay. Tobacco and tomato suspension cells were maintained in MS media, as described previously (2, 3). The alkalinization assay has been described previously (2). Briefly, cells were used for assay 3–5 days after transfer. One milliliter of cells was aliquoted into each well of 24-well cell culture cluster plates (Corning) and allowed to equilibrate on an orbital shaker at 160 rpm for 1 h. Aliquots (1–10 μl) of fractions to be assayed were added to the cells, and the alkalinization of the medium was monitored with time by using an Orion Model EA940 pH meter with an Orion semimicro pH electrode (Orion, Boston).

Polypeptide Isolation. Tobacco plants were harvested for extraction 4 weeks after planting. Approximately 120 plants were defoliated, and the leaves were frozen in liquid N_2 and stored at -20°C. The frozen leaves were crushed to a fine powder and homogenized with 500 ml of 1% trifluoroacetic acid (TFA) for 2 min in a 1-liter Waring blender. The mixture was filtered through eight layers of cheesecloth and then one layer of Miracloth (Calbiochem). The filtrate was clarified by centrifugation at 10,000 $\times$ *g* for 20 min at 2°C.

The supernatant from the centrifugation was loaded onto a 40-μm 3 $\times$ 25-cm C18 reversed-phase flash column (Fischer), previously equilibrated with 0.1% TFA/H_2O. Compressed nitrogen was used at 8 psi to elute the extract. After sample loading, the column was washed with 0.1% TFA/H_2O, and the retained material was eluted with successive washes of 10 and 50% methanol in 0.1% TFA. The 50% methanol-eluting fraction had strong activity in alkalinating the media of tobacco suspension cells. The methanol was removed by using a rotary evaporator, followed by lyophilization to dryness. The yield was 330 mg.

Forty milligrams of the lyophilized powder was dissolved in 1 ml of 0.1% TFA in water and injected into a semipreparative reversed-phase C18-HPLC column (Vydac, Hesperia, CA). After 2 min, a 90-min gradient from 0 to 40% acetonitrile/0.1% TFA was used. The flow rate was 2 ml/min, and 2-ml fractions, monitored at 220 nm, were collected. Ten microliters from each fraction was assayed with the tobacco suspension-cultured cells, as described above. The active peak, eluting at 77 and 78 min from 8 individual separations (320 mg total), was pooled and lyophilized. The lyophilized material was dissolved in 1 ml 5 mM potassium phosphate, pH 3, in 25% acetonitrile and clarified by centrifugation. The solution was chromatographed by using a polySULFOETHYL Aspartamide column strong cation exchange-HPLC (The Nest Group, Southport, MA). A 60-min gradient was applied from 0 to 100% 5 mM potassium phosphate, 500 mM potassium chloride, pH 3, in 25% acetonitrile. The flow rate was 1 ml/min, and the elution profile was monitored at 214 nm. One-milliliter fractions were collected, and a 5-μl aliquot from each fraction was assayed for alkalinating activity. Fractions 50–51 were pooled and lyophilized.

The lyophilized material was dissolved in 1 ml of 0.1% TFA in water and chromatographed on a narrow-bore reversed-phase C18-HPLC column (Vydac) previously equilibrated with 0.1% TFA in water. After a 2-min wash, a 90-min gradient was applied from 0 to 100% methanol/0.05% TFA. The flow rate was 0.25 ml/min, and the elution profile was monitored by absorbance at 214 nm. Fractions were collected at 1-min intervals. Alkalinization activity was assayed by using 2 μl from each fraction and was

Abbreviations: TFA, trifluoroacetic acid; MALDI, matrix-assisted laser desorption ionization; alkRALF, alkylated RALF polypeptide; MAP, mitogen-activated protein; EST, expressed sequence tag.

*Present address: Department of Biological Sciences, University of South Carolina, Columbia, SC 29208.

†To whom reprint requests should be addressed. E-mail: cabudryan@hotmail.com.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

PLANT BIOLOGY

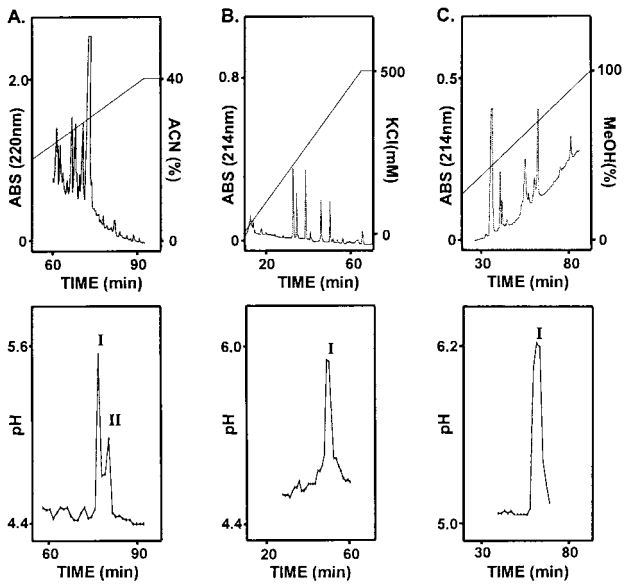

Fig. 1. Purification of RALF by HPLC. Activity was determined by adding 2–10 μl of each fraction from HPLC separations to 1 ml of tobacco cells and measuring the pH of the media after 15 min (*Lower*). (*A*) Semipreparative C18 HPLC purification. Fractions 77–78 were pooled and lyophilized. (*B*) Strong cation exchange HPLC purification. Fractions 50–51 were pooled and lyophilized. (*C*) Narrow-bore C18 HPLC purification. Fractions 61–63 were pooled and lyophilized.

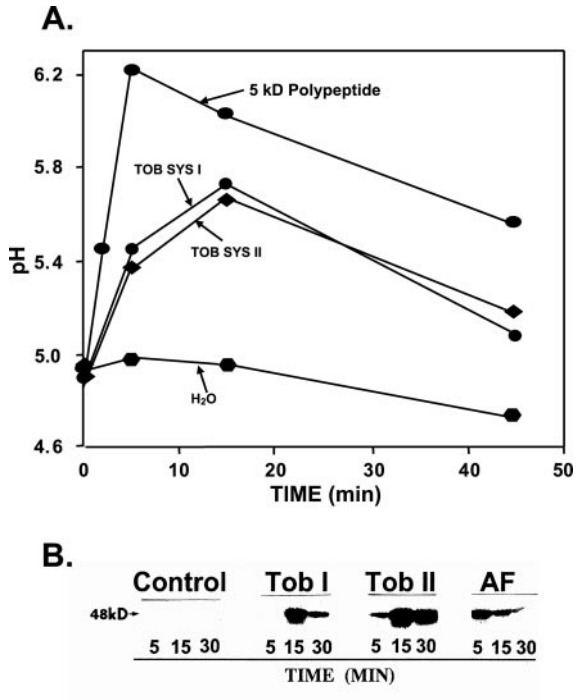

Fig. 2. Biological activities of the 5-kDa polypeptide from tobacco leaves. (*A*) A comparison of the alkalinization of tobacco suspension-cultured cell medium by the synthetic 5-kDa alkalinating factor with two tobacco systemins (2). Each polypeptide was assayed at a concentration of 1 nM. (*B*) MAP kinase activity in tobacco suspension-cultured cells treated with the 5-kDa polypeptide (AF) compared with the activity induced by two tobacco systemins. [Reproduced with permission from ref. 2 (Copyright 2001, Macmillan Magazines Ltd.).]

found in fractions 61–63. These fractions were pooled and used for amino acid sequence analysis and matrix-assisted laser desorption ionization (MALDI)-MS analysis. MALDI-MS analyses were performed by using a PerSeptive Biosystems (Foster City, CA) Voyager Biospectrometry Workstation. N-terminal sequencing was performed by Edman degradation on an Applied Biosystems Model 475 sequencer by using the manufacturer's protocol.

The synthetic tomato polypeptide was synthesized by using 9-fluorenylmethyl chloroformate solid-phase chemistry with a peptide synthesizer (Applied Biosystems Model 431A) with *p*-methyl benzyhydrylamine resin according to the manufacturer's protocol.

After synthesis, 20 mg of reduced synthetic polypeptide was oxidized by dissolving in 100 ml of degassed 0.1 M ammonium bicarbonate and incubating for 2 days in an opened flask, then lyophilized. The oxidized products were separated on strong cation exchange-HPLC, as described above. Four peaks eluted, two major and two minor, detected by UV absorbance at 214 nm. The alkalinization assay using suspension-cultured *Lycopersicon peruvianum* cells (2) determined that activity was present only in the first major peak that eluted. This peak from several repetitions was pooled, lyophilized, and further purified by semiprep C18-HPLC, as described above.

The synthetic polypeptide that contained activity was digested with trypsin to determine the disulfide bridging of the active polypeptide. The polypeptide was dissolved in 50 mM ammonium bicarbonate at a concentration of 10 pmol/μl, and 10 μl was added to 1 pmol of trypsin and allowed to digest overnight at room temperature. Tryptic peptides were analyzed by MALDI-MS.

Alkylation of Half-Cystines in RALF. The disulfide bridges of the active, synthetic RALF polypeptide were reduced and alkylated as follows: 2 mg of polypeptide (0.4 μmols) was dissolved in 1 ml 0.1 M ammonium bicarbonate, and then 12 μmols of DTT was added. The solution was incubated for 40 min at 50°C in the dark, cooled to room temperature, and 6 μmols (6 μl of a 1 M stock) iodoacetamide was added. After 40 min at room temperature in the dark, the reaction was terminated by acidification with TFA. The reduced alkylated RALF polypeptide (alkRALF) was separated from reactants by C18-HPLC. Mass change analysis of the reduced alkylated polypeptide by MALDI-MS confirmed the presence of four carboxyamidomethyl groups in the polypeptide.

Mitogen-activated protein (MAP) kinase activity was assayed as described previously (4).

```
   GCACCAGGGAAGTTTCAACAGCGAAAACATAGTAAAAAGATACCAAAAACCAGCGGTCCA
   AAGCATAAACAGGCAGAAATAGCAAAAGGGTGTTTTAATTTCTCATCATTTTTTCAGTAC
   AAGGTAAAAATGGGAGTTCCTTCAGGTTTGATTCTTTGTGTTCTGATCGGAGCTTTTTTC
1          M  G  V  P  S  G  L  I  L  C  V  L  I  G  A  F  F   - 17

   ATTTCAATGGCGGCGGCCGGAGATAGTGGGGCCTACGATTGGGTGATGCCGGCGAGATCT
18 I  S  M  A  A  A  G  D  S  G  A  Y  D  W  V  M  P  A  R  S   - 37

   GGTGGGGGATGCAAAGGGAGTATCGGAGAGTGCATTGCTGAAGAAGAGGAGTTTGAGCTG
38 G  G  G  C  K  G  S  I  G  E  C  I  A  E  E  E  E  F  E  L   - 57

   GACAGTGAGTCAAACAGGCGCATTTTAGCCACCAAAAAGTACATCAGCTATGGTGCACTG
58 D  S  E  S  N  R  R  I  L  A  T  K  K  Y  I  S  Y  G  A  L   - 77

   CAGAAGAACAGTGTACCTTGTTCTCGCCGTGGAGCTTCGTATTACAACTGCAAACCTGGT
78 Q  K  N  S  V  P  C  S  R  R  G  A  S  Y  Y  N  C  K  P  G   - 97

   GCTCAGGCTAATCCTTACTCTCGTGGATGCAGTGCTATCACTCGTTGCAGGAGTTAAGTT
98 A  Q  A  N  P  Y  S  R  G  C  S  A  I  T  R  C  R  S  *      -115

   CTGAATTTCTCTTCTTCTTCCACAGAATCCAAAAATATTGAATTTTTCATGGAAAATTGA
   AT
```

Fig. 3. The nucleotide and deduced protein sequences of RALF precursor cDNA. The RALF polypeptide sequence is at the C terminus of the preproprotein and is underlined. Arrow indicates the predicted signal peptide cleavage site.

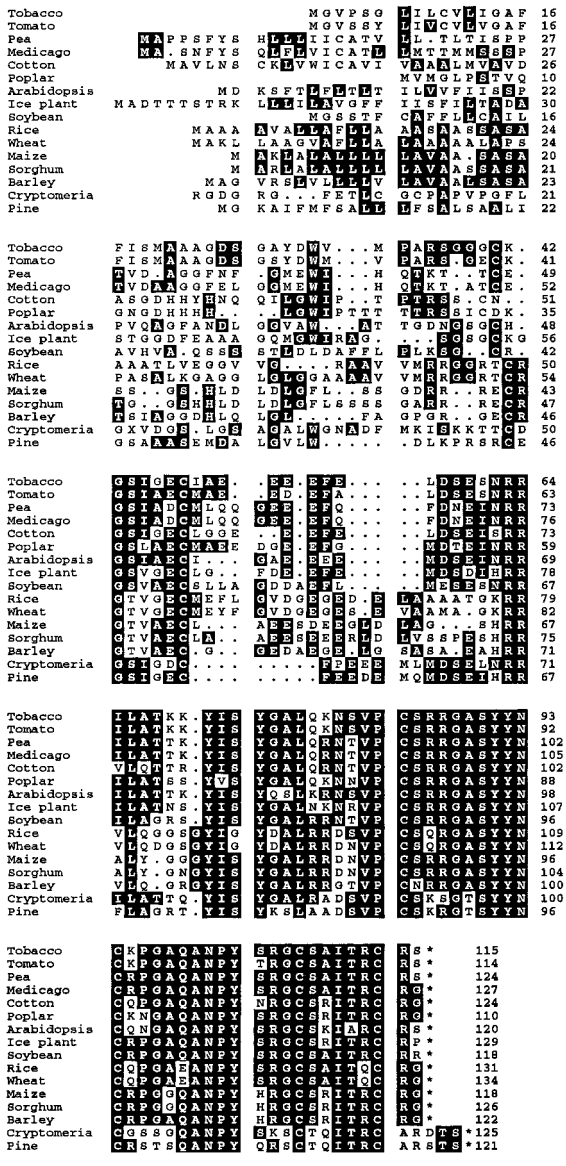

Fig. 4. The amino acid sequence of tobacco RALF compared with known EST sequences.

Table 1. Identity of tobacco RALF preproprotein with some of the ESTs available at GenBank

Species	Library source	% identity
Tomato	Callus	87 (101/115)
Tomato	Fruit	87 (101/115)
Tomato	Ovary	87 (101/115)
Tomato	Leaf	86 (100/115)
Tomato	Root	67 (74/110)
Ice plant	Leaf	60 (72/119)
Cotton	Fiber	59 (65/109)
Pea	Seedling	58 (67/115)
Medicago	Seedling root	63 (63/99)
Medicago	Leaf	71 (59/83)
Arabidopsis	Root	60 (65/108)
Arabidopsis	Seedling	70 (64/91)
Arabidopsis	Rosette	80 (60/75)
Soybean	Hypocotyl	57 (63/110)
Soybean	Leaf	81 (61/75)
Soybean	Root	56 (62/110)
Hybrid aspen	Cambial region	80 (60/75)
Potato	Leaf	74 (57/77)
Barley	Spike	48 (58/119)
Sorghum	Rhizome	50 (61/120)
Pine	Xylem	49 (54/109)
Pine	Polen cone	53 (61/113)
Rice	Plant	60 (51/85)
Rice	Panicle	52 (48/91)
Rice	Root	54 (47/87)
Maize	Endosperm	49 (59/120)
Cryptomeria	Inner bark	65 (48/73)
Wheat	Spike	49 (50/101)

Results and Discussions

We recently developed a cell suspension culture assay that can identify polypeptide hormones in plant extracts that interact with cell surface receptors and cause alkalinization of the medium (2). Such interactions can be assayed by monitoring the pH of cell culture medium caused by a polypeptide–receptor interaction (2–6). This assay was used recently to identify and isolate two tobacco systemins (2). During the purification of the tobacco systemins, alkalinization assays of the eluting fractions from the 50% methanol wash from C18-HPLC (Fig. 1*A Upper*) revealed the presence of an additional strong alkalinating activity (Fig. 1*A*

Tomato and *Arabidopsis* Seedling Experiments. Tomato (*Lycopersicon esculentum* cv Castlemart II) and *Arabidopsis thaliana* (Columbia ecotype) seeds were surface sterilized by using 70% (vol/vol) ethanol for 30 sec and 15% (vol/vol) commercial bleach with 0.1% (vol/vol) Tween 20 for 20 min. After sterilization, the seeds were washed five times in sterile water and were dispersed in water to germinate. The germination was performed in 250-ml Erlenmeyer flasks under 16-h days with 150 $\mu E \cdot m^{-2} \cdot s^{-1}$ (E, Einstein; 1 E = 1 mol of photons) light intensity, while agitated on a shaker at 100 rpm (Model G-10, New Brunswick Scientific). After 2 days, germinating tomato seeds were transferred to 50-ml flasks with MS media [MS salts (GIBCO/BRL)/B5 vitamins (Sigma)/30 g/liter sucrose, pH 5.8] containing 10 μM RALF or 10 μM alkRALF or an equal volume of water. *Arabidopsis* seeds were germinated in 50-ml flasks for 1 day and transferred to 12-well tissue culture plates containing MS media as above with 10 μM RALF or 10 μM alkRALF or an equal volume of water. All solutions were filter-sterilized (0.2-μm pores), and the seedlings were photographed 48 h after being transferred to the media.

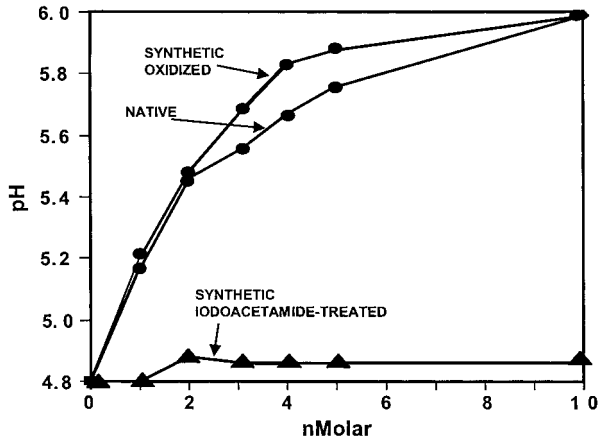

Fig. 5. Comparison of the alkalinization response of tomato suspension cells with native, synthetic, and alkylated RALF. The pH responses of the synthetic peptides were compared with native tomato RALF after 10 min.

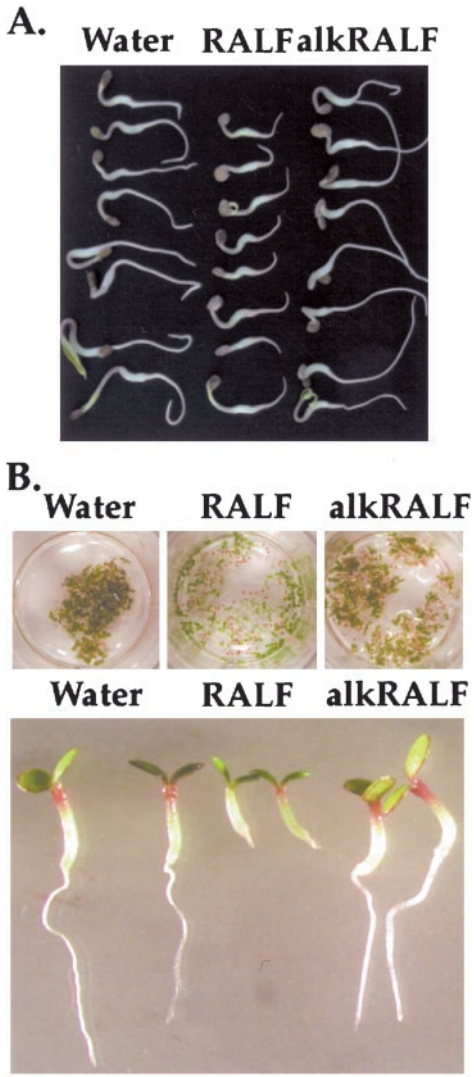

Fig. 6. Effects of water, 10 μM RALF, and 10 μM alkRALF on germinating seedlings. Seeds were germinated in water for 2 days and then treated as above for 2 days. (*A*) Tomato seedlings. A severe arrest of root growth and development can be seen in tomato seedlings treated with RALF. (*B*) *Arabidopsis* seedlings treated as in *A*. Photos are of *Arabidopsis* seedlings taken at different magnifications.

Lower, peak I). The component was further purified by using SCX-HPLC (Fig. 1*B Upper* and *Lower*) and narrow-bore C18-HPLC (Fig. 1*C Upper* and *Lower*). Peak II (Fig. 1*A Lower*) is an isoform of RALF (data not shown). Purification of Peak I resulted in a single polypeptide species with a mass of 5,332.7 Da, determined by MALDI-MS. A partial N-terminal sequence of the tobacco leaf polypeptide was determined to be $^{+}NH_3$—ATKKYISYGALQKNSVP—.

The polypeptide caused a stronger and more rapid alkalinization of tobacco suspension-cultured cells than the two tobacco systemins (Fig. 2*A*) and, like the tobacco systemins, it induced the activation of a MAP kinase in the tobacco cells (Fig. 2*B*) but, similar to the alkalinization assay, the MAP kinase activity peaked earlier than the activity induced by either of the tobacco systemins. Because of the rapid response in the alkalinization assay, we called the polypeptide RALF. RALF did not appear to be a defensive wound signal, in that it did not induce the synthesis of tobacco trypsin inhibitor in leaves of tobacco plants, as do tobacco systemins (2).

To identify a full length tobacco RALF cDNA, a tomato expressed sequence tag (EST) (EST262422) containing a sequence identical to the N-terminal amino acids of tobacco RALF was used as a probe to screen a tobacco leaf cDNA library. The deduced ORF in the isolated tobacco cDNA coded for a preproprotein of 115 amino acids that harbored the RALF sequence at the C terminus (Fig. 3). A signal peptide cleavage site is predicted between residues 23 and 24 (7). An -Arg-Arg- motif is present just two residues upstream from the N terminus of the mature RALF, suggesting that, as with animal and yeast polypeptide prohormones, a proteolytic enzyme with a dibasic substrate specificity site may be involved in the processing of RALF from its precursor. If so, this would be the first example of a processing role for such an enzyme in plants. Another dibasic amino acid motif is found within the RALF sequence, between Cys-18 and -28. This dibasic pair may be an additional processing site or may serve a role in degrading and inactivating the polypeptide after the initial processing and signaling have taken place, or it may have no regulatory role at all. A kexin-like enzyme has been associated with turnover of systemin (8).

RALF is unlike any other plant polypeptide hormone reported to date in having highly conserved homologs throughout the plant kingdom. In Fig. 4 is shown the amino acid sequence of the tobacco RALF precursor compared with ESTs that have been reported from numerous plant species. Although the N-terminal 40–50 amino acids do not exhibit high identities, the region just N-terminal to the RALF sequence and RALF itself exhibit a high percentage of identity. The homology in primary structure found among widely divergent plant species suggests that the RALF sequence has been highly conserved over millions of years and that RALF has a role of fundamental importance in many plant families. The ESTs in Fig. 4 were derived from a variety of tissues including roots, shoots, leaves, and flowers (Table 1). Thus, the expression of the RALF precursor gene does not appear to be associated with any particular plant cell types, suggesting that it may be involved in some basic physiological role that is common to different aspects of growth and development.

By using the same methodologies described in *Materials and Methods* for the purification of tobacco RALF, polypeptides were identified in crude preparations obtained from tomato and alfalfa leaves that had elution characteristics similar to the tobacco polypeptide. The N-terminal amino acid sequence of tomato RALF was determined to be $^{+}NH_3$—ATKKYISYGALQKN—, with a mass of 5,346.8 Da. This is 14 mass units larger than tobacco RALF, which could be accounted for by the substitution of a tobacco RALF Ser with a Thr in tomato RALF (see Fig. 4). The N-terminal sequence determined for alfalfa RALF was $^{+}NH_3$—ATTKYISYGALQRNTVPXSRRGASYYN—, with a mass of 5,375.9 Da. All three purified RALFs were capable of producing an alkalinization response with tomato, tobacco, and alfalfa cells (data not shown).

Tomato RALF was chemically synthesized and, after oxidation and disulfide bridge formation, one species was as active as native RALF in the alkalinization assay (Figs. 2*A* and 5), indicating that the polypeptide can refold to its native form. Two disulfide bridges were identified in the active RALF polypeptide that occurred between Cys-18 and -28 and between Cys-41 and -47. These results indicated that the oxidation of RALF resulted in the formation of an active RALF with the correct disulfide bridges. Some of the reoxidized polypeptides crosslinked abnormally, and none of these forms exhibited activity. Reduction of the disulfides of synthetic active RALF and alkylation of the free sulfhydryls with iodoacetamide rendered the polypeptide inactive. The activities of synthetic oxidized RALF, alkRALF, and native RALF are compared in Fig. 5. The half-maximal concentrations of native and synthetic RALF in the alkalinization assays are less than 2 nanomolar, which is near the half-maximal

activity required for systemin induction of defense genes (0.5 nanomolar) and is in the same range in which many animal hormones activate physiological processes.

The initial biological activities found for tobacco RALF were its potent ability to cause alkalinization of tobacco cell suspension culture medium and its induction of MAP kinase activity in the cultured cells. We sought clues to its possible role in plants by supplying the polypeptide to excised plants and to germinating seeds and observing its effect on the growth and development of the seedlings. Whereas supplying excised tomato plants with RALF provided no clues to its function, RALF did cause a striking arrest of the growth of newly developing roots emerging from tomato seeds (Fig. 6*A*). The effects of tomato RALF on the elongation of roots of newly germinating *Arabidopsis* seeds were also assayed. In Fig. 6*B*, the inhibitory effects of RALF on root growth and development of *Arabidopsis* seedlings are shown. With both tomato and *Arabidopsis* seedlings, the roots were much shorter than roots of seedlings treated with water or with the inactive alkRALF. Additionally, cotyledons of *Arabidopsis* seedlings treated with RALF exhibited a lighter green color compared with controls, perhaps reflecting the underdeveloped roots, which may have limited the uptake of nutrients from the medium. Visual inspection of the roots of *Arabidopsis* RALF-treated plants indicated that the elongation zones and meristems were arrested, and that the meristem cells appeared to be somewhat enlarged (data not shown). There appeared to be an absence of root hair on the stunted roots. When returned to media without the presence of RALF, the roots resumed growth (data not shown).

Nine RALF isoforms have been identified in the *Arabidopsis* genome (GenBank, accession nos. bab01857, al161537, aaf98428, u78721, z97339, bab00062, aaf02876, aaf64534, and bab1o941). ESTs have been identified in many different organs and tissues (see Table 1), indicating that the polypeptide may have other roles in plants beyond the arrest of root growth that is described here.

Until 1991, plant hormone signaling was thought to be mediated only by small hormonal signals not found in animals or yeast. In 1991, an 18-aa polypeptide, called systemin, was found to regulate systemic wound signaling (9). Systemin, which is derived from a 200-aa precursor, prosystemin (10), is released on wounding during herbivore and pathogen attacks and interacts with a specific receptor on tomato cell surface membranes (3, 11) to activate a signal transduction pathway that regulates defense gene expression (12). The binding of a defense polypeptide signal in plants raised the question of whether plants may use other polypeptide hormones to regulate defensive or developmental processes. Since 1991, several other polypeptide signals have been reported in plants that include two 18-aa glycosylated systemins from tobacco (2), phytosulfokines (13), small sulfated 4- to 5-aa polypeptides that regulate cell proliferation and differentiation (14), CLAVATA3 (15), a 73-aa polypeptide from *Arabidopsis* meristems with a role in development (16), and 55- to 58-aa cystine-rich polypeptides (SCRs) that regulate self-incompatibility in *Brassica* (17, 18).

We now describe herein another signaling polypeptide, RALF, that was isolated from tobacco, tomato, and alfalfa leaves. The RALF cDNA showed that, like other hormones, it is derived from a preproprotein. EST searches indicate that the RALF gene is highly conserved and is likely ubiquitous in plants with important regulatory roles. Although the polypeptide appears to severely arrest root growth and development in tomato and *Arabidopsis*, its biochemical and physiological mechanisms in regulating this process, and perhaps other processes in plants, remain to be determined. RALF provides the newest example of polypeptide signaling in plants and increases the anticipation that many more polypeptides will be identified in the near future that regulate defensive and developmental processes in plants.

We thank Sue Vogtman for growing plants for this research, Dr. Gerhard Munske for amino acid sequencing and peptide synthesis, and Dr. William Siems for MALDI-MS analyses. This research was supported by Project 1791 of the College of Agriculture and Home Economics, by the National Science Foundation, Grant IBN 0090766, and by the Charlotte Y. Martin Foundation.

1. Ryan, C. A. & Pearce, G. (2001) *Plant Physiol.* **125,** 65–68.
2. Pearce, G., Moura, D. S., Stratmann, J. & Ryan, C. A. (2001) *Nature (London)* **411,** 817–820.
3. Scheer, J. M. & Ryan, C. A. (1999) *Plant Cell* **11,** 1525–1535.
4. Stratmann, J. W. & Ryan, C. A. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 11085–11089.
5. Felix, G. & Boller, T. (1995) *Plant J.* **7,** 381–389.
6. Schaller, A. & Oecking, C. (1999) *Plant Cell* **11,** 263–272.
7. Nielsen, H., Ingelbrecht, J., Brunak, B. & von Heijne, G. (1997) *Protein Eng.* **10,** 1–6.
8. Schaller, A. & Ryan, C. A. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 11802–11806.
9. Pearce, G., Strydom, D., Johnson, S. & Ryan, C. A. (1991) *Science* **253,** 895–897.
10. McGurl, B., Pearce, G. Orozco-Cardenas, M. & Ryan, C. A. (1992) *Science* **255,** 1570–1573.
11. Meinkl, T., Boller, T. & Felix, G. (1998) *Plant Cell* **10,** 1561–1570.
12. Ryan, C. A. (2000) *Biochim. Biophys. Acta* **1477,** 112–121.
13. Matsubayashi, Y. & Sakagami, Y. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 7623–7627.
14. Matsubayashi, Y. & Sakagami, Y. (2000) *J. Biol. Chem.* **275,** 15520–15525.
15. Fletcher, J. C., Brand, U., Running, M. P., Simon, R. & Meyerowitz, E. M. (1999) *Science* **283,** 1911–1914.
16. Clark, S. E., Williams, R. W. & Meyerowitz, E. M. (1997) *Cell* **89,** 575–585.
17. Schopfer, C. R., Nasrallah, M. E. & Nasrallah, J. B. (1999) *Science* **286,** 1697–1700.
18. Stein, J. C., Howlett, B. H., Boyes, D. C., Nasrallah, M. E. & Nasrallah, J. B. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 8816–8820.